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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

We have been testing the hypothesis that chronic alcohol exposure alters expression and/or function of kainate receptors (KA-Rs) in the hippocampus. These receptors have been shown to control hippocampal excitability and an ethanol-induced upregulation of KA-Rs could contribute to the hyperexcitability associated to alcohol withdrawal. We have exposed adult male rats to a liquid diet containing ethanol for 16 days. We have euthanized these rats on the last day of this diet at the peak of ethanol consumption and removed their brains. At this point, we have completed an initial assessment of the effects of chronic ethanol exposure on the expression of KA receptor subunits and other glutamate receptor subunits in whole hippocampi homogenates by using Western blot techniques. These initial experiments did not detect a change in the expression levels of any of these glutamate receptor subunits. We are currently analyzing results of autoradiographical and immunohystochemical studies, which have greater sensitivity than Western immunoblotting studies, to assess ethanol's effects in hippocampal subregions. Electrophysiological studies of KA receptor-mediated currents in hippocampal subregions are also underway. Importantly, we will soon initiate studies with alcohol withdrawn animals, which will likely have changes in KA receptors as suggested by experiments with cultured neurons.

14. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award)
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INTRODUCTION:

Alcohol-related medical disorders affect many organs and systems of the body, including the central nervous system (CNS). As with other drugs of abuse, long-term alcohol ingestion results in the development of tolerance, addiction, and dependence. Alcohol produces these effects by altering the actions of neurotransmitters and their receptors in the brain. Chronic ethanol exposure has complex and long-lasting effects on the function and/or expression of a myriad of neurotransmitter receptors and their modulators. A group of proteins affected by chronic ethanol exposure are ligand-gated ion channels such as the glutamatergic ionotropic receptors. Glutamate activates three major classes of ionotropic receptors. These three major types of channels are the NMDA, AMPA and kainate receptors (KA-Rs). The purpose of this proposal is to test whether or not chronic ethanol exposure results in alterations in subunit expression and/or function of KA-Rs in the hippocampus. Maladaptive changes in hippocampal KA-R expression could contribute to the pathophysiology of alcohol withdrawal syndrome. The alcohol withdrawal syndrome is associated with neuronal hyperexcitability and seizures. Since kainate receptors are important regulators of excitability in the hippocampus, upregulation of these receptors may have an important role in the pathophysiology of alcohol withdrawal syndrome.

BODY:

Our overall strategy is to perform studies that assess KA-R expression and function in parallel. Western blot, radioligand binding and immunohistochemical experiments are being used to determine the effects of chronic ethanol exposure and withdrawal on the expression levels of these receptors. Patch-clamp electrophysiological experiments with hippocampal slices are being used to determine the functional consequences of chronic exposure to ethanol and withdrawal. Our specific objectives are:

Objective #1: To determine whether chronic ethanol exposure results in a change in expression of kainate receptors. We are measuring [^{3}H] vinylidene-kainate binding to hippocampal tissue sections from control, chronically ethanol-treated rats and ethanol-withdrawn rats. We are also assessing specific changes in kainate receptor subunit expression. Specifically, we have measured levels of GluR5, GluR6/7 and KA2 subunits in hippocampal homogenates by using Western blot techniques and will be also assessing KA-R subunit levels in hippocampal tissue sections by using immunohistochemical techniques. These immunohistochemical experiments will determine specific hippocampal neuronal populations where kainate receptors are affected by chronic ethanol exposure and/or withdrawal.

Objective #2: To determine whether chronic ethanol exposure results in changes in the function of pre- and postsynaptic kainate receptors in rat hippocampal CA1 and CA3 pyramidal neurons. We are using whole cell patch-clamp electrophysiological methods to determine the effects of chronic ethanol exposure and withdrawal on synaptic and agonist-evoked kainate currents. We are also measuring effects on presynaptic kainate receptor-mediated inhibition of evoked excitatory and inhibitory synaptic currens in rat hippocampal CA1 and CA3 pyramidal neurons.

The following **Statement of Work** was proposed to complete these objectives:

Year #1:

We will perform Western blot and radioimmunohystochemistry experiments, quantify, and interpret the results of these experiments. We estimate that we will be able to complete experiments with anti-GluR6/7 antibodies during the first year. We will also initiate the electrophysiological characterization of kainate receptor-mediated synaptic and evoked currents in the CA3 region of the hippocampus. We will present our preliminary findings at a scientific meeting.

Year #2:

We will continue Western blot and radioimmunohystochemistry experiments, quantify, and interpret the results of these experiments. We estimate that we will be able to complete experiments with anti-GluR6/7 antibodies and initiate studies with anti-GluR5 antibodies during the second year. We will complete the electrophysiological characterization of kainate receptor-mediated synaptic and evoked currents in the CA3 region of the hippocampus. We will initiate the electrophysiological characterization of kainate receptor function in the CA1 region of the hippocampus, including experiments on kainate receptor-mediated regulation of GABA release. We will present our preliminary findings at a scientific meeting.

Year #3:

We will finish Western blot and radioimmunohystochemistry experiments, quantify, and interpret the results of these experiments. We will complete experiments with anti-GluR5 antibodies and with anti-KA2 antibodies during the last year. We will also complete electrophysiological experiments of kainate receptor function in the CA1 region of the hippocampus, including experiments on kainate receptor-mediated regulation of GABA release. We will present our preliminary findings at a scientific meeting. We will submit a paper to a peer-review scientific journal reporting the findings of our study.

Research Accomplishments:

Western Blot Studies:

We are pleased to report that we have made excellent progress with this part of project during the first year of funding and that we are ahead of schedule. We were originally planning to complete experiments with anti-GluR6/7 antibodies during the first year and to perform experiments with anti-GluR5 and anti-KA2 antibodies during years 2 and 3, respectively. However, we were able to complete during the first year the entire Western blot experiments with anti-GluR5, anti-GluR6/7 and anti-KA2 antibodies in hippocampal homogenates from control and chronic ethanol-exposed animals. Moreover, we extended these studies to other glutamate receptor subunits (GluR1, GluR2/3, NR1, NR2A, NR2B, NR2C, NR1-N1, NR1-C1, and NR1-C2). Quantitative studies on the expression of these 12 glutamate

receptor subunits were performed with hippocampal homogenates from rats that were exposed to a 16-day alcohol diet that yields blood alcohol levels between 0.24 g/dl (legal intoxication limit in most states is 0.08 g/dl). For this set of studies, rats were euthanized at the peak of ethanol consumption to prevent alcohol withdrawal. In a separate group of rats, Drs. Savage and Valenzuela determined that removal of the ethanol-containing diet results in the development of alcohol withdrawal syndrome, which indicates that it causes ethanol dependence. Unexpectedly and contrary to other published reports, the expression levels of these subunits was not affected by long-term alcohol exposure in these homogenates. We presented our findings at the Society for Neurosciences Meeting held in November 2000 (see appendix). Moreover, a paper reporting the findings of this study is currently in press in the peer-reviewed journal *Alcoholism: Clinical and Experimental Research* (see appendix). Over the next two years, we will concentrate on characterizing the effect of chronic ethanol-exposure on KA-R levels by using immunohistochemistry and receptor autoradiography.

Immunohistochemistry and Receptor Autoradiography:

These studies will provide information on KA-R expression in hippocampal sub-regions. This approach should detect more subtle changes in receptor expression than our Western immunoblotting studies with whole-hippocampal homogenates. At this point, we have completed a long-term alcohol diet and have frozen the brains from 10 control and 10 ethanol exposed rats. As for the Western immunoblotting studies, these brains were obtained from rats euthanized on the last day of the 16-day ethanol diet at the peak of ethanol consumption to prevent ethanol withdrawal. Last month, we prepared sagittal sections from these frozen brains by using a cryostat. Half of these sections were used to perform KA-R autoradiographical studies using [^{3}H] vinylidene-kainate as the ligand for these studies. We just finished exposing these sections to X-ray film and are in the process of quantifying the results of these experiments by using the ImagePro computer program. We should complete this quantification over the next 2-3 months. Preparation of the brain sections and these receptor autoradiography studies are being performed in the laboratory of the co-investigator, Dr. Savage.

The other half of these sections was used for immunohistochemical staining in the P.I.'s laboratory. We immunostained these sections with anti-GluR6/7 antibodies. It should be emphasized that we have refined our immunohistochemical experiments with respect to those originally proposed in our grant application. We originally proposed to use radiolabeled secondary antibodies and autoradiography to detect binding of primary antibodies to our tissue sections. However, the University of New Mexico-School of Medicine recently acquired a state-of-the-art confocal microscope. The availability of this microscope is now allowing us to perform quantitative immunofluorescence measurements instead of the radioimmunohistochemical experiments originally proposed. Because of its high resolution, confocal microscopy has emerged as technique of choice to quantify receptor expression in immunolabeled tissues. Optimal illumination, along with the ability to scan samples in all axis, make the laser scanning confocal microscope (LSCM) an ideal tool for measuring receptor levels in immunohistochemical studies. Moreover, imaging illumination, scanning and acquisition parameters are computer-controlled, which makes it relatively straightforward to standardize such parameters for the analysis of multiple samples. In addition, images acquired with a LSCM can be subsequently processed with computer software to accurately determine immunofluorescence intensity levels for specific neurotransmitter receptor subunits. Good et al. were the first to apply confocal microscopy to the quantification of receptor levels in single cells (Good et al., 1992). These investigators

demonstrated that epidermal growth factor receptor density could be determined as accurately with confocal microscopy techniques as with ^{125}I-EGF binding techniques. LSCM was subsequently used by Dodge et al. to quantify intracellular levels of a Clara cell secretory 10 kD protein in rat bronchi (Dodge et al., 1994). More recently, Gazzaley et al. used LSCM to quantify the regulation of NMDAR1 subunit protein expression by estradiol in the rat hippocampus (Gazzaley et al., 1996b; Gazzaley et al., 1996a). At this point, we are in the process of acquiring confocal microscopy images of the sections from control and chronic ethanol treated rats that we recently immunostained with anti-GluR6/7. We will start quantitating the results of these measurements by using the ImagePro computer program over the next 2 months. We should complete this quantification over the next 6 months.

Slice electrophysiological studies:

This proposal also includes a component in which the function of glutamate receptors is being analyzed by electrophysiological techniques in brain slices from control and alcohol exposed rats. Dr. Weiner from Wake Forest University is in charge of these experiments and is making excellent progress with these experiments. The main goal of these experiments is to determine the effect of chronic ethanol exposure on pre- and postsynaptic kainate receptor function in rat hippocampal CA1 and CA3 pyramidal neurons. Our previous work has shown that specific populations of kainate receptors in the hippocampus are acutely inhibited by ethanol. We hypothesized that chronic exposure to ethanol (or withdrawal from this exposure) would lead to an upregulation of kainate receptor function within the hippocampus and that this increased kainate receptor activity might contribute significantly to the hyperexcitability associated with ethanol exposure and withdrawal.

The kainate subclass of glutamate receptors exerts a number of pre- and postsynaptc roles in the mammalian CNS. During the time period preceding the funding of this award, we and others have continued to characterize the physiology and pharmacology of kainate receptors in the hippocampus and other brain regions. From these studies, it has become increasingly apparent that presynaptic kainate receptors may play a more dominant physiological role than their postsynaptic counterparts. To that end, we have decided to focus our initial experimental efforts on Experiment 3 of the original proposal. In this experiment, we sought to determine if chronic ethanol exposure and withdrawal upregulates presynaptic kainate receptors at inhibitory GABAergic synapses in the rat hippocampal CA1 region. These synapses are gated by $GABA_A$ receptors and mediate the vast majority of inhibitory synaptic transmission in the mammalian central nervous system. Moreover, there is compelling evidence that ethanol intoxication is mediated, at least in part, by an enhancement of GABAergic synaptic transmission. Many studies have also demonstrated that activation of kainate receptors potently inhibits evoked $GABA_A$ receptor-mediated inhibitory postsynaptic currents (IPSCs) in this brain region. We have shown that acute exposure to ethanol significantly reduces the activity of these presynaptic kainate receptors. This results in an upregulation of inhibitory GABAergic tone, consistent with the known effects of ethanol in this brain region. In this first experiment, we sought to determine the following:

1. Does chronic ethanol exposure result in an adaptive upregulation in presynaptic kainate receptor function.

2. Does chronic ethanol exposure result in tolerance to the acute effect of ethanol on presynaptic kainate receptor function or the acute effect of ethanol on inhibitory $GABA_A$ IPSCs.

In this first study, rats were exposed to a sixteen-day liquid diet regimen as outlined in the original application. Control subjects were pair-fed an isocaloloric diet that did not contain ethanol. The protocol was designed such that one subject would complete the treatment period on each experimental day. In total, 10 ethanol treated and 10 control subjects were used in this first study. There were no significant differences in initial weight or weight gain between these two groups throughout this treatment period (Figure 1). On the morning of sacrifice, brain ethanol levels were sampled from both groups of animals. The mean brain ethanol concentration of the experimental group was 49.4 ± 3.6 mM. No significant ethanol levels were detected in the control group.

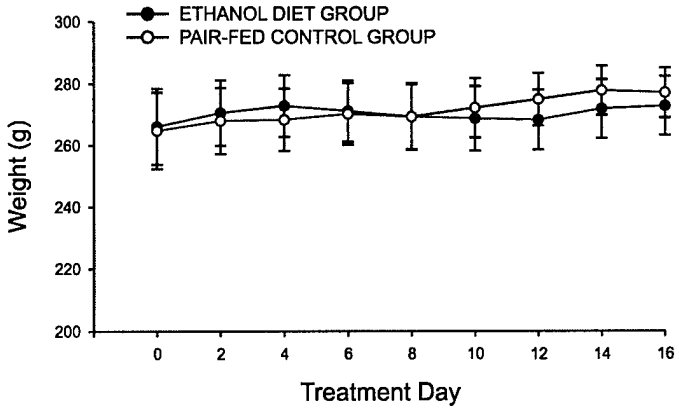

Figure 1. Effect of Liquid Diet on Rat Body Weight.

In this first study, hippocampal slices were prepared as described in the original proposal and slices were incubated in normal artificial cerebrospinal fluid for at least two hours and no more than eight hours prior to recording. This protocol was employed to determine if there was any effect of acute *in vitro* withdrawal from ethanol on the electrophysiological parameters to be assessed. In total, we recorded from 36 neurons from ethanol-treated subjects and 34 neurons from controls. There were no significant differences in the initial resting membrane potential (Ethanol = -72.3 ± 1.5 mV; Control = -71.9 ± 1.2 mV) or input resistance (Ethanol = 143 ± 13 MΩ; Control = 146 ± 14 MΩ) between these two groups of neurons.

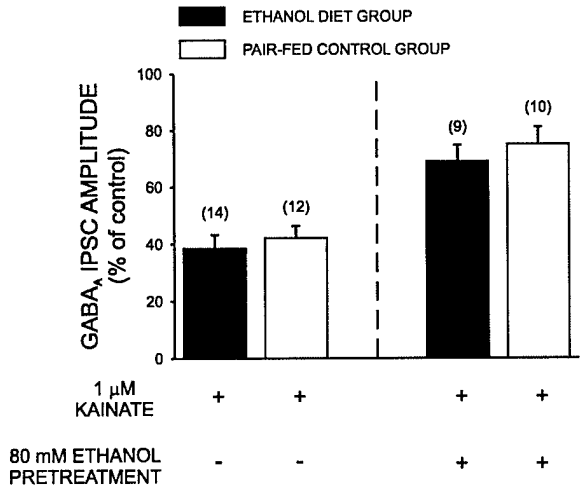

Figure 2. Bar graph summarizing the effect of chronic ethanol exposure on the inhibitory effect of 1 μM kainate on $GABA_A$ IPSCs recorded from rat hippocampal CA1 neurons. Recordings were carried out in the absence and presence of 80 mM ethanol as indicated in the graph. Note that chronic ethanol treatment had no effect on kainate inhibition of $GABA_A$ IPSCs or the antagonism of this effect by acute exposure to ethanol.

In the first experiment, CA1 pyramidal cells were voltage-clamped at –30 mV and $GABA_A$ IPSCs were evoked every 20 seconds in the presence of 50 μM APV and 1 μM NBQX to block NMDA and AMPA receptors respectively. 5 mM QX-314 was included in the recording pipette solution to block $GABA_B$ receptors. We next tested the effect of bath application of 1 μM kainate on the amplitude of $GABA_A$ IPSCs recorded in the absence

and presence of 80 mM ethanol. Although kainate significantly inhibited the amplitude of $GABA_A$ IPSCs, no difference was observed between the effect of kainate on the two experimental groups. (Figure 2). Bath application of 80 mM ethanol potentiated $GABA_A$ IPSCs to a similar extent in both groups of slices. Moreover, ethanol pretreatment significantly reduced the inhibitory effect of kainate to the same extent in slices from both treatment groups (Figure 2).

The results of this initial study suggest that chronic ethanol exposure does not result in a significant upregulation of presynaptic kainate receptor function at GABAergic synapses in the rat hippocampal CA1 region. In addition, we observed no significant tolerance to the either the acute potentiating effect of ethanol on $GABA_A$ IPSCs or the acute inhibitory effect on kainate at these synapses. These data suggest that, although the activity of both GABAergic synapses and presynaptic kainate receptors are significantly modulated by acute ethanol exposure, these targets do not seem to be altered by chronic exposure to this drug. It should be noted however that this experiment was carried out on slices prepared from animals sacrificed immediately following their last exposure to ethanol. It remains to be determined if alterations in either $GABA_A$ or kainate receptor function may be observed after one to two days of withdrawal from ethanol treatment.

We are currently initiating an experiment to test the effect of 24 and 48 hours of ethanol withdrawal following our chronic exposure paradigm on presynaptic kainate receptor function at GABAergic synapses. Once these experiments are completed, we will examine the effects of chronic ethanol exposure and withdrawal on postsynaptic kainate receptor function in the CA1 and CA3 region of the rat hippocampus as outlined in the original proposal.

In addition to the studies described, there are two related ongoing projects in our laboratory that have been partly funded by this grant. The first involves the characterization of the physiological role and ethanol sensitivity of kainate receptors in the rat nucleus accumbens. The nucleus accumbens is a brain region that is intimately involved in motivation, attention and reward. Although this brain region receives substantial glutamatergic innervation from many limbic structures, the role of kainate receptors within this nucleus is unknown. We have recently demonstrated that functional pre- and postsynaptic kainate receptors are present in the nucleus accumbens and that some of these receptors are inhibited by ethanol. The second project involves the characterization of a novel interaction between ethanol and presynaptic $GABA_B$ receptors in the rat hippocampus. Activation of presynaptic $GABA_B$ receptors inhibits GABA release in the hippocampus and many other brain regions. We have recently demonstrated that, at GABAergic synapses in the rat hippocampal CA1 region, ethanol significantly potentiates presynaptic $GABA_B$ receptor activity. This interaction effectively reduces ethanol's overall potentiating effect on GABAergic transmission at these synapses. Further experiments are planned to further characterize these novel acute effects of ethanol and to determine if they are affected by chronic ethanol exposure and withdrawal.

Plans for Years 2 and 3:

After completing the receptor autoradiographical and immunohistochemical experiments mentioned above on the effects of chronic ethanol on KA-R expression, we will concentrate our efforts on the study of the KA-R expression in the hippocampi of ethanol withdrawn rats using these experimental

approaches. Recent experiments with cultured hippocampal neurons performed in the P.I.'s laboratory suggest that KA-R expression and function is indeed upregulated 24 hrs after ethanol withdrawal. Therefore, we believe that alcohol withdrawal will also upregulate these receptors in hippocampi from ethanol withdrawn rats. We will initiate these studies during the first half of the year 2002. Dr. Weiner will continue to concentrate his efforts on characterizing the function of pre- and post-synaptic KA-R in the CA1 and CA3 regions of the hippocampus of ethanol withdrawn rats.

KEY RESEARCH ACCOMPLISHMENTS:

- Completed Western immunoblotting quantification of GluR5, GluR6/7 and KA2 subunits in hippocampal homogenates from control and chronic ethanol-exposed rats (tissue collected at peak of ethanol consumption). Quantification of NMDA and AMPA receptor subunits was also performed. Findings are currently in press in a peer-reviewed journal (see below and appendix).
- [^{3}H] vinylidene kainate binding experiments in tissue sections from 10 control and 10 chronically ethanol exposed rats have been performed. X-ray autoradiography just completed. Quantification of results currently underway (expected to be completed over the next 2-3 months).
- Immunostainning with anti-GluR6/7 antibodies in tissue sections from 10 control and 10 chronically ethanol exposed rats have been performed. Confocal microscopy acquisition of images currently underway. Quantification of results will be completed over the next 6 months.
- Electrophysiological characterization of the effects of long-term ethanol exposure on presynaptic KA-Rs at inhibitory GABAergic synapses in CA1 region of the hippocampus.

REPORTABLE OUTCOMES:

Peer-reviewed article (see appendix):

Ferreira, V.M., Frausto, S. Browning, M.D., Savage, D.D., Morato, G.S., and **Valenzuela, C.F.** Ionotropic Glutamate Receptor Subunit Expression in the Rat Hippocampus: Lack of an Effect of a Chronic Exposure Paradigm. *Alcoholism. Clin. Exp. Res.* In Press.

Bookchapters:

Valenzuela, C. F. and Savage, D.D. Quantitative analysis of ethanol's effects on neurotransmitter receptor expression. In: Methods for Alcohol-Related Neuroscience Research- Liu and Lovinger, Editors. CRC Press. *In Press.*

Weiner J. L. Electrophysiological Assessment of Synaptic Transmission in Brain Slices. In: Methods for Alcohol-Related Neuroscience Research- Liu and Lovinger, Editors. CRC Press. *In Press.*

Meeting Presentations:

C. F. Valenzuela, V.M. Ferreira, M.D. Browning, G.S. Morato. Long-term alcohol exposure and hippocampal ionotropic glutamate receptor subunit expression. Poster presented at the Society for

Neurosciences Meeting. New Orleans, LA. November 4-9, 2000 (see appendix).

C. F. Valenzuela. Introduction to glutamatergic mechanisms in the pathophysiology of substance abuse. Oral presentation. Winter conference on Brain Research. Streamboat Springs, CO. January 20-26, 2001.

J.L. Weiner. Glutamatergic mechanisms in the pathophysiology of substance abuse. Oral presentation. Winter conference on Brain Research. Streamboat Springs, CO. January 20-26, 2001.

O.J. Ariwodola and **J.L. Weiner**. Ethanol potentiation of GABAergic synaptic transmission in the rat hippocampus may be self-limiting. Presented at the Research Society on Alcoholism Meeting, Montreal, 2001.

T.L. Crowder, T.L. and **Weiner, J.L.** Ethanol inhibits presynaptic kainate receptors at glutamatergic synapses in the rat nucleus accumbens. Presented at the Research Society on Alcoholism Meeting, Montreal, 2001.

CONCLUSIONS:

We are making excellent progress with this project. We have just published a paper reporting our initial characterization by Western immunoblot of the effects of long-term ethanol exposure on the expression levels of KA-R and other glutamatergic subunits (see appendix). These studies with whole hippocampal homogenates did not reveal any changes in the expression levels of these subunits; however, we are currently assessing expression levels in subregions of the hippocampus by using higher resolution techniques (i.e. receptor autoradiography and immunohistochemistry-confocal microscopy). Experiments performed in our laboratory with cultured hippocampal neurons suggest that KA-R subunit expression levels change after ethanol withdrawal and, therefore, we are currently investigating whether this effect can also be observed in the hippocampus of animals undergoing ethanol withdrawal. Functional studies are currently underway to determine if the function of pre- and post-synaptic KA-R is impaired in these animals as well.

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Good MJ, Hage WJ, Mummery CL, De Laat SW, Boonstra J (1992) Localization and quantification of epidermal growth factor receptors on single cells by confocal laser scanning microscopy. J Histochem Cytochem 40:1353-1361.

APPENDICES:

Ferreira, V.M., Frausto, S. Browning, M.D., **Savage, D.D.**, Morato, G.S., and **Valenzuela, C.F.** Ionotropic Glutamate Receptor Subunit Expression in the Rat Hippocampus: Lack of an Effect of a Chronic Exposure Paradigm. *Alcoholism. Clin. Exp. Res.* In Press.

C. F. Valenzuela, V.M. Ferreira, M.D. Browning, G.S. Morato. Long-term alcohol exposure and hippocampal ionotropic glutamate receptor subunit expression. Poster presented at the Society for Neurosciences Meeting. New Orleans, LA. November 4-9, 2000.

ABSTRACTS

SOCIETY FOR NEUROSCIENCE

30TH ANNUAL MEETING • NEW ORLEANS, LA. • NOVEMBER 4–9, 2000

26

Part 1

PART 1 OF 2

EXCITATORY AMINO ACID RECEPTORS: LIGAND-GATED CHANNELS III

425.1

DISTRIBUTION OF GLUTAMATE RECEPTORS IN EXPERIMENTALLY INDUCED CORTICAL MALFORMATIONS. G. Hagemann[1*], H.J. Luhmann[2], M. Kluska[1], C. Redecker[1], O.W. Witte[1]. [1]*Dept Neurology, Heinrich-Heine-University, 40225 Duesseldorf, Germany,* [2]*Institute of Neurophysiology, Heinrich-Heine-University, 40225 Duesseldorf, Germany*

Cortical malformations comprise a heterogeneous group of disturbances of cortical development which are increasingly recognized in association with epilepsy, mental retardation, motor disabilities, and/or neuropsychological deficits, such as dyslexia. Experimental studies on the freeze lesion model of cortical malformations reveal widespread alterations in cortical connectivity and glutamatergic hyperexcitability. Receptor autoradiography of glutamate and GABA receptors demonstrated an imbalance of excitation and inhibition. Although electrophysiological data on the role of the GABA-ergic system is controversial, recent immunohistochemical data showed widespread alterations of GABA(A) receptor subunit distribution (Redecker et al., J. Neurosci., in press). This study aimed to determine the distribution of the main glutamate receptors NR1, NR2A/B, GluR2,3 (AMPA), and GluR5,6,7 (Kainate) using conventional immunohistochemical techniques. Within the microgyral area all receptors displayed a typical staining pattern reflecting the infolding of layers I to III without any major change in histological or cellular distribution. In contrast to previous electrophysiological and receptor autoradiographical observations no remote effects of receptor subunit expression could be detected immunohistochemically. This result suggests a significant role of modulation of glutamate receptor binding properties rather than receptor redistribution in this model of developmental malformations. *Supported by: grants from Deutsche Forschungsgemeinschaft SFB 194 (HJL and OWW) and from the HHU to GH.*

425.2

GLUTAMATE RECEPTOR SUBUNIT EXPRESSION IN PRIMARY NEURONAL AND SECONDARY GLIAL CULTURES. A.S.J. Lesage*, N. Janssens. *CNS Discovery Research, Janssen Research Foundation, Beerse, Belgium*

We have investigated the expression of the following ionotropic glutamate receptor subunits in primary (nearly pure) neuronal cultures from cortex, hippocampus and cerebellum: NR1, NR2A, B, C, D (NMDA receptor), GluR1, 2, 3, 4 (AMPA receptor), GluR5, 6, 7 and KA1, 2 (kainate receptor). We also document the expression of the metabotropic glutamate receptors mGluR1, 2, 3, 4, 5, 6, 7, 8 in the neuronal cultures and in astroglial cultures grown in serum versus defined media (containing amongst others EGF). The presence of RNA was determined via RT-PCR using subunit specific primers. Protein expression was investigated via Western blot analysis. We found that the NMDA receptor subunits NR1, NR2A and NR2B were expressed in all 3 cultures. NR2C and NR2D were not detected. Each of the 3 cultures also showed expression of the 4 AMPA receptor subunits GluR1-4. RT-PCR detected the RNA of all the kainate subunits, but Western blots showed expression of only GluR6 and KA2 protein. GluR5 and GluR7 were not detectable, and we lacked an antibody for KA1. The expression of mGluR proteins in neuronal cultures indicated the presence of mGluR1, 2/3, 4 and 5 in all cultures. Although a PCR signal was present for mGluR6 and 8 in the cultures, mGluR8 protein was not detected, and we could not verify mGluR6 protein, due to the lack of an antibody. mGluR7 protein was expressed in hippocampal and cortex cultures only. In astroglial cultures grown in serum containing media, only mGluR5 protein was found. When the astrocytes were cultured in defined medium, mGluR1, 5 and mGluR8 were detected.

425.3

ENRICHMENT OF SYNAPTIC PROTEINS IN RAT CORTICAL AND CEREBELLAR POSTSYNAPTIC DENSITIES. R.A. Al-Hallaq*, R.P. Yasuda, B.B. Wolfe. *Dept Pharmacology/Interdisciplinary Prog Neurosci, Georgetown Univ Medical Center, Washington, DC, USA*

The postsynaptic density (PSD) contains receptor, signaling, and scaffolding proteins that must be selectively localized and anchored at the PSD to allow for proper response to presynaptic stimulation. With respect to the N-methyl-D-aspartate receptor (NMDAR), NR1 subunit splice variants containing the C1 cassette have been suggested to be critical for proper insertion into the membrane [Ehlers, et al.,(1995) Science 269:1734-1737], a step crucial to proper localization to the PSD. Furthermore, it has been suggested that synaptic NMDAR complexes contain the NR2A subunit, while NR2B subunits may be confined to an extrasynaptic population of NMDAR [Stocca, G & S Vicini(1998) J Physiol 507(1):13-24]. Based on these studies, we tested whether NMDAR C1-containing NR1 subunits and NR2A subunits were more highly localized to the synapse relative to crude preparations. Western blot analysis was used to determine the level of enrichment of these subunits in PSDs prepared from rat cortex and cerebellum relative to crude cortical and cerebellar homogenates, respectively. Levels of enrichment of the NMDAR subunits NR2A, NR2B, and the various NR1 cassettes were determined with specific antibodies. We also measured the fold enrichment of subunits of other receptor proteins, such as $GABA_A$ β and AMPAR GluR1, as well as PSD-associated proteins, such as PSD-95, Chapsyn-110, SAP102, and CaMKII-α. Preliminary data suggest that NR2A and NR2B subunits are equally enriched in the PSDs, whereas NMDAR NR1-C1 is more highly enriched in cerebellar but not cortical PSDs. The other proteins measured do not show differing levels of enrichment. Supported by NS36246, AG08206.

425.4

LONG-TERM ALCOHOL EXPOSURE AND HIPPOCAMPAL IONOTROPIC GLUTAMATE RECEPTOR SUBUNIT EXPRESSION. C.F. Valenzuela[1*], V.M. Ferreira[1], M.D. Browning[2], G.S. Morato[3]. [1]*Neurosci, U. of New Mexico HSC, Albuquerque, NM, USA,* [2]*Pharmacology, U. of Colorado HSC, Denver, CO, USA,* [3]*U. of Santa Catarina, Florianopolis, Brazil*

Long-term exposure to ethanol has been shown to result in compensatory changes in the expression levels of glutamate-gated ion channel subunits. These maladaptive changes in subunit expression have been postulated to contribute to the neurochemical imbalance associated with the alcohol withdrawal syndrome. In this study, we examined the effects of a 16-day ethanol diet on the expression of subunits belonging to the NMDA, AMPA and kainate families of ionotropic glutamate receptors in the rat hippocampus. We found that this diet induced alcohol dependency as indicated by a 13-parameter withdrawal scale and audiogenic seizure sensitivity. We assessed expression of NR1, NR2A, NR2B, NR2C, GluR1, GluR2/3, GluR5, GluR6/7, and KA2 subunits by using western immunoblotting techniques at day 16 of the diet before alcohol withdrawal. We did not find statistical significant changes in the expression of any of these subunits in hippocampal homogenates from ethanol-exposed rats. We also examined expression of NR1 subunits containing the N1, C1 and C2 cassettes and only found a small trend towards a decrease in the expression levels of those subunits containing the N1 cassette. These results indicate that maladaptive changes in the levels of glutamate ionotropic receptor subunit expression migh not always take place after long-term alcohol exposure. Studies are underway to determine if expression of these subunits is altered after alcohol withdrawal. *Supported by: Supported by NIH grant AA00227, US Army Contract #322015, and by CNPq, Brazil.*

425.5

EFFECTS OF DEAFFERENTATION ON THE EXPRESSION OF AMPA-TYPE GLUTAMATE RECEPTORS IN THE RAT SUPERIOR COLLICULUS. A. Laudanna*, L.R.G. Britto. *Physiol.Biophys., Univ.São Paulo, São Paulo, Brazil*

To understand the participation of the glutamatergic system in visual processing, we evaluated the contribution of retinal and cortical afferents to the pattern of the AMPA-type glutamate receptor immunoreactivity in the rat superior colliculus (SC). Adult Lister hooded rats were subjected to combined unilateral retinal and cortical ablations (one retina/contralateral cortex) under ketamine/xylazine anesthesia. Immunohistochemical experiments were performed on SC sections after different survival times (2-15 days). Antisera against AMPA receptor subunits GluR1 and GluR2/3 (Chemicon, Temecula, CA, USA) and appropriate secondary antisera (Jackson Labs., West Grove, PA, USA) were used with standard avidin-biotin-peroxidase protocols. In agreement with previous data, GluR1-positive neurons and neuropil were found in the superficial layers of SC, and GluR2/3-positive neurons were observed in the intermediate layers. After deafferentation, the immunostaining for GluR1 and GluR2/3 was increased in the deafferented side in comparison to the control side within short survival periods (up to 5 days). After longer survival times, immunostaining for both antisera appear to decrease. The glutamatergic denervation thus produced biphasic alterations on the expression of the AMPA-type glutamate receptor subunits in SC glutamateceptive neurons, suggesting that both the retinal and cortical innervation regulate the expression of those proteins in the SC. *Supported by: FAPESP, CNPq, PRONEX/MCT (Brazil).*

425.6

CHANGES IN AMPA RECEPTOR ANTAGONIST BINDING AND SUBUNIT PROTEIN IMMUNOSTAINING IN BENZODIAZEPINE-TOLERANT RAT. B.J. VanSickle*, S.M. Lilly, E.I. Tietz. *Department of Pharmacology, Medical College of Ohio, Toledo, OH, United States of America*

Studies of excitatory amino acid receptors (EAARs) revealed regulation of AMPA receptor (AMPAR) subunit mRNA in hippocampal regions in flurazepam (FZP)-treated (100 mg/kg X 3 days; 150 mg/kg X 4 days, p.o.) rats. A 28% increase in AMPAR-mediated miniature (m)EPSCs amplitude in CA1 pyramidal cells suggested that changes in EAAR structure and function may contribute to development or expression of BZ tolerance. To assess changes in AMPAR structure, total receptor number was determined in FZP-treated and control Sprague-Dawley rats using a near-saturating concentration (25 nM) of the AMPAR antagonist [^{3}H]Ro48-8587. Rats were euthanized 2 days following 1-week oral FZP when BZ tolerance is present. Autoradiographic images of 20 μm sections were taken with NIH Image software and calibrated with [^{3}H]thymidine standards. Significant increases in specific binding were found in stratum pyramidale of area CA1 (+20.7%, p=0.03) and CA2 (+27.0%, p=0.03), as well as the dentate granule cell layer (+23.4%, p=0.05). AMPAR subunit protein was analyzed using quantitative immunotechniques. Western analysis of microdissected hippocampus was unable to detect significant alterations in GluR1 or GluR2 subunit protein, whereas small (-5 to -7%), but significant ($p<0.05$), decreases in GluR2 subunit immunostaining were found in dentate gyrus. Failure to detect changes in CA1 AMPAR subunit proteins, despite increases in [^{3}H]Ro48-8587 binding, suggests the immunotechniques may not be sensitive to localized changes in AMPARs. Increased [^{3}H]Ro48-8587 binding suggests an increased AMPAR number which may underlie the increased AMPAR-mediated mEPSC amplitude in the CA1 region. *Supported by: NIDA grant R01-DA04075.*

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Ionotropic Glutamate Receptor Subunit Expression in the Rat Hippocampus: Lack of an Effect of a Long-Term Ethanol Exposure Paradigm

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Background: Studies have shown that acute ethanol exposure inhibits ionotropic glutamate receptor function and that long-term ethanol exposure results in maladaptive increases in the expression of some of these receptors in neurons. It has been postulated that these changes, when unopposed by ethanol, contribute, in part, to the hyperexcitability associated with ethanol withdrawal. In this study, we compared the effect of long-term ethanol exposure on the hippocampal expression levels of subunits belonging to the three families of ionotropic glutamate receptors.

Methods: Adult male Sprague-Dawley rats were fed an ethanol-containing diet for 16 days. This diet contained 0% ethanol on days 1 and 2, 3% on days 3 and 4, 5% on days 5 to 7, and 6.7% on days 8 to 16. Control rats received an equivalent amount of an isocaloric diet without ethanol. Rats were killed on day 16 at the peak of ethanol consumption. Hippocampal homogenates were prepared by sonication and analyzed by Western immunoblotting techniques. On a separate group of rats, we measured withdrawal scores and audiogenic seizures on day 17.

Results: Ethanol-exposed rats had significantly higher withdrawal scores, and a significantly higher percentage of them developed audiogenic seizures; this indicates that the 16-day ethanol diet induces ethanol dependence. Unexpectedly, we found that expression of NR1 (including the expression of NR1 subunits containing the N1, C1, and C2 inserts), NR2A, NR2B, NR2C, GluR1, GluR2/3, GluR5, GluR6/7, and KA2 subunits was not altered in hippocampal homogenates from ethanol-exposed rats.

Conclusions: These results indicate that maladaptive changes in the hippocampal expression levels of ionotropic glutamate receptor subunits do not always occur in ethanol-dependent rats. Consequently, other mechanisms must mediate the hyperexcitability state associated with ethanol withdrawal in these animals.

Key Words: Chronic, Alcohol, NMDA, AMPA, Kainate.

THE SUPERFAMILY OF glutamate-gated ion channels mediates the majority of excitatory transmission in the mammalian central nervous system and is composed of the NMDA, AMPA, and kainate receptors. Multiple polypeptide subunits have been identified for each of these glutamate receptor subfamilies. Six NMDA receptor (NMDA-R) subunits (NR1, NR2A, NR2B, NR2C, NR2D, and NR3A), four AMPA receptor subunits (GluR1, GluR2, GluR3, and GluR4), and five kainate receptor subunits (GluR5, GluR6, GluR7, KA1, and KA2) have been characterized to date [for a review, see Ozawa et al. (1998)]. Three exons of the NR1 subunit mRNA can be differentially spliced. They encode 21 to 38 amino acid sequences located in the N-terminus domain (termed the N1 cassette) or the C-terminus domain (termed the C1 and C2 cassettes) [for a review, see Zukin and Bennett (1995)]. These NMDA-R subunits and NR1 alternatively spliced variants can form channels with diverse subunit compositions and functional properties. The expression levels of these subunits are precisely regulated by complex signal transduction processes, and changes in ionotropic glutamate receptor subunit expression levels have been shown to have important roles, not only in normal development and synaptic plasticity, but also in pathophysiological conditions.

Long-term exposure to ethanol has been shown to induce maladaptive changes in the protein levels of glutamate-gated ion channel subunits [reviewed in Valenzuela and Harris (1997)]. Western blot studies detected increases in NR1 protein levels in brain synaptosomes (Chen et al., 1997) and in hippocampal homogenates (Devaud and Morrow, 1999; Trevisan et al., 1994) from rats chronically exposed to ethanol. Another study found increases in NR1, NR2A, and NR2B protein levels in homogenates from the

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cortex and hippocampus of rats exposed to intragastric infusions of ethanol for 6 days (Kalluri et al., 1998). An increase in hippocampal NR1 and NR2A subunit levels was reported by Snell et al. (1996) in mice exposed to an ethanol-containing liquid diet for 2 weeks. Taken together, the results of these studies with chronically ethanol-exposed animals suggest that NMDA-R subunit expression is altered by long-term ethanol exposure. It has been postulated that these maladaptive changes in NMDA-R subunit expression, when unopposed by ethanol, contribute, in part, to the neurotransmission imbalance associated with the ethanol withdrawal syndrome.

Much less is known about the effects of long-term ethanol exposure of animals on the expression of non–NMDA-R subunits. One study reported that a 12-week exposure to ethanol did not change the levels of GluR1 and GluR2 subunits in the rat hippocampus (Trevisan et al., 1994). However, the effect of long-term ethanol exposure on the expression of other non–NMDA-R subunits in the mammalian brain remains an open question. In this study, we examined the effects of a 16-day ethanol diet on the expression of subunits belonging to the NMDA, AMPA, and kainate families of glutamate receptors. We concentrated our studies on the rat hippocampus because ionotropic glutamate receptors have been shown to have important roles in seizure generation in this brain region (Ben-Ari and Cossart, 2000; Kullmann et al., 2000)

METHODS

Chronic Ethanol Exposure Paradigm

All animal experiments were authorized by the University of New Mexico Health Sciences Center–Institutional Animal Care and Use Committee and performed in accordance with its guidelines. Rats were maintained in the Animal Resource Facilities at the University of New Mexico School of Medicine and allowed to acclimate to the rat room environment for at least 1 week before initiation of the diet described below. Adult male Sprague-Dawley rats weighing approximately 250 g were individually housed in a room maintained at 22°C on an 8-hr dim light/16-hr dark cycle (lights on from 0900 to 1700 hr). Rats received a Bioserv (Frenchtown, NJ) chocolate-flavored liquid diet that was based on the Lieber-DeCarli formulation (Lieber and DeCarli, 1982). This liquid diet provides 1 kcal/ml. A group of rats (ethanol-treated group) was offered 80 ml of diet containing 0% v/v (days 1–2), 3% v/v (days 3–4), 5% v/v (days 5–7), and 6.7% v/v ethanol (days 8–16). Rats consumed approximately 80 ml/day during days 1 to 4, approximately 60 to 70 ml/day during days 5 through 7, and approximately 40 to 65 ml/day during days 8 to 16. A control group of rats was given equivalent amounts of the liquid diet without ethanol; their diet was made isocaloric to the ethanol-containing diet by the addition of maltose-dextrins. The liquid diet was available to the rats during the 16-hr dark cycle (starting at 1700 hr), and water bottles were removed from the cages during this period. The liquid diet tubes were removed at 0900 hr each morning and the water bottles returned to the cages. Tubes were removed in the morning because rats are nocturnal animals that do not consume significant amounts of this liquid diet during the light cycle (Savage et al., unpublished data, 2001). Rats had free access to water during the light hours to prevent dehydration. Diet consumption patterns were checked daily. We did not detect statistically significant differences in body weight between the ethanol and control groups (Fig. 1). To confirm that this diet induced ethanol dependence, we determined baseline withdrawal scores on a separate group of rats by using the scale described by

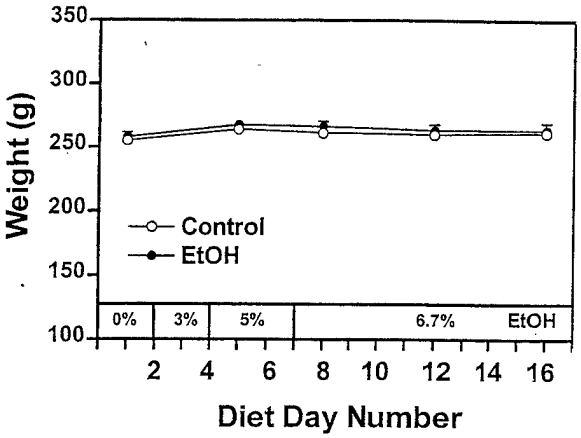

Fig. 1. Shown is the weight of chronically ethanol (EtOH)-treated rats and control rats. Values were not found to be statistically different by two-way ANOVA ($p > 0.3$; $n = 8$ rats per group).

Lal et al. (1988) (Table 1). In this group of rats, we also measured audiogenic seizures. Individual rats were placed in a circular chamber (40 cm in diameter × 60 cm high). A sound stimulus (108 dB relative to 2×10^{-4} dynes/cm^2) was generated by an electric bell mounted on the ceiling of the chamber. The sound stimulus was initiated 15 sec after the rat was placed into the chamber and was continued until the onset of convulsion or for a maximum of 90 sec if no convulsion was observed. Convulsions in these rats were characterized by a running phase, followed by clonic and, in most cases, tonic convulsions. Data are given as the percentage of animals tested per diet group that developed seizures.

Rats were killed by rapid decapitation without anesthesia. They were euthanized at the peak time (2200–2330 hr) of ethanol consumption on day 16 of the ethanol treatment. After death, blood was obtained from the heart into ethylenediaminetetraacetic acid–containing tubes, and blood ethanol levels were measured with a kit based on the activity of the enzyme alcohol dehydrogenase. At this time point, blood ethanol levels ranged from 50 to 80 mM. After decapitation, brains were rapidly removed and hippocampi dissected out on ice. Hippocampi were homogenized by sonication in phosphate-buffered saline plus a protease inhibitor cocktail (P-8340, Sigma Chemical Co., St. Louis, MO). The protein content of the homogenates was determined by Lowry assay. Samples were diluted to 2

Table 1. Baseline Withdrawal Scores Obtained on Day 17 at 1530 hr (see "Methods" for more details) on a Separate Group of Rats

Parameter scored	Group	
	Control	Ethanol
General activity	0.5 ± 0.2	0.7 ± 0.1
Shakes, jerks, and twitching	0.0	0.3 ± 0.3
Head tremor	0.5 ± 0.2	1.0 ± 0.3
Vocalization	0.6 ± 0.3	2.1 ± 0.2*
Avoidance	1.0 ± 0.2	1.7 ± 0.3*
Rigidity of axial muscles by palpation	1.2 ± 0.1	1.7 ± 0.1*
Tail tremor	1.2 ± 0.1	1.5 ± 0.2
General tremor	1.0 ± 0.0	1.8 ± 0.1*
Motor task	1.1 ± 0.1	2.1 ± 0.2*
Bracing posture	1.1 ± 0.1	1.5 ± 0.2*
Convulsions	1.0 ± 0.0	1.1 ± 0.1
Tail-lifting tremor	0.6 ± 0.3	2.2 ± 0.2*
Grooming	0.6 ± 0.2	1.8 ± 0.3*
Average scores	0.8 ± 0.1	1.5 ± 0.1**
Audiogenic seizures	0%	80%

Scores were obtained as described by Lal et al. (1988). Values represent mean ± SEM of five rats per treatment group.

* $p < 0.05$ and ** $p < 0.01$ by t test.

Shown at the end of the table is the percentage of these rats that developed audiogenic seizures.

to 4 mg/ml with phosphate-buffered saline, mixed with 2× SDS-PAGE sample buffer, boiled for 2 min, and stored in aliquots at −20°C.

Western Immunoblotting

Samples (10–30 μg of total protein per lane) were separated on 7.5% polyacrylamide minigels and electrotransferred to nitrocellulose membranes. Nonspecific binding of antibodies to nitrocellulose membranes was prevented by blocking with a solution containing 10% nonfat dry milk and 0.4% Tween-20 (ICI Americas, Inc., Wilmington, DE), plus 0.01% sodium azide. Blots were analyzed with a chemiluminescence assay kit by following the manufacturer's instructions (Roche Biochemicals, Indianapolis, IN). Membranes were then probed with rabbit anti–NR1-N1 insert, anti–NR1-C1 insert, anti–NR1-C2 insert, anti-NR2A, anti-NR2B, and anti-NR2C antibodies (all produced, purified, and characterized in MDB's laboratory). Membranes were also probed with rabbit anti-GluR6/7, anti-KA2, anti-GluR1, and anti-GluR2/3 antibodies from Upstate Biotechnology (Lake Placid, NY) and with goat anti-GluR5 or anti-NR1 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit and anti-goat immunoglobulin G-peroxidase conjugates were obtained from Roche Biochemicals. Densitometric analyses of Western blot chemiluminescence x-ray films were performed by using an Image-Pro® Plus image analysis system (Media Cybernetics, L.P., Silver Spring, MD). In all cases, a protein standard curve was generated from a single batch of hippocampal tissue homogenate kept at −20°C. This standard curve was included in the same membrane (by using 15-well combs) as samples from control and chronic ethanol-treated animals. By using linear regression analysis, this curve was used to calculate relative units of subunit protein concentrations in samples from control and ethanol-exposed animals (Chandler et al., 1999). Tubulin levels (anti–β-tubulin monoclonal antibody from Sigma) were measured in all samples, and relative units of subunit protein were divided by the relative units of tubulin in each sample to normalize for differences in gel loading. Tubulin levels were unaffected by the long-term ethanol exposure paradigm used in this study (control, 17.2 ± 1.2, and ethanol-exposed, 16.7 ± 1.2 densitometry arbitrary units; $n = 8$).

RESULTS

To confirm that this diet induced ethanol dependence, we determined withdrawal scores by using a modified version of a previously described scale (Table 1) (Lal et al., 1988). These determinations were performed with a separate group of animals that were not used for Western blot analyses. The parameters listed in Table 1 were scored exactly as described by Lal et al. (1988) by two observers that were blind to the treatment group assignment of each rat. On day 17 at 1530 hr, we observed statistically significant differences between control and ethanol-treated rats in several of the scored parameters, including vocalizations, general tremor, motor task, tail-lifting tremor, and grooming (Table 1). A trend toward an increase was also found in other parameters, such as avoidance, rigidity of axial muscles by palpation, head tremor, and bracing posture (Table 1). Average scores were significantly increased in the ethanol-treated animals (Table 1). We also assessed the development of audiogenic seizures in this group of animals. None of the five rats from the control group developed audiogenic seizures (Table 1). Conversely, four of five animals in the chronically ethanol-exposed group developed audiogenic seizures (Table 1). Taken together, these results clearly indicate that our diet-exposure paradigm induces ethanol dependence. It should be emphasized, however, that we performed these studies only to establish that the ethanol-exposure paradigm produced ethanol dependence in our hands and that our studies of the effect of ethanol on hippocampal glutamate receptor expression were not aimed at determining the mechanism of ethanol withdrawal–induced audiogenic seizures; the neuronal network for the generation of these seizures is primarily contained in certain brainstem structures [reviewed in Faingold et al. (1998)].

Western blot analyses were performed to assess expression of NMDA, AMPA, and kainate hippocampal receptor subunits during the last day of the diet-exposure paradigm. For these studies, rats were killed on day 16 at the peak of ethanol consumption. We initially studied expression of NMDA-R subunits and did not detect a significant effect of chronic ethanol exposure on NR1, NR2A, NR2B, or NR2C subunit levels (Fig. 2). Moreover, we did not detect significant effects of long-term ethanol exposure on expression levels of NR1 subunits containing the N1, C1, or C2 cassettes (Fig. 3).

We also measured expression of non–NMDA-R subunits. We assessed expression of AMPA receptor subunits with anti-GluR1 and anti-GluR2/3 antibodies. We did not detect significant effects of chronic ethanol exposure on the expression levels of these subunits in hippocampal homogenates (Fig. 4). Expression levels of kainate receptor subunits were determined with anti-GluR5, -GluR6/7, and -KA2 antibodies. As with NMDA and AMPA receptor

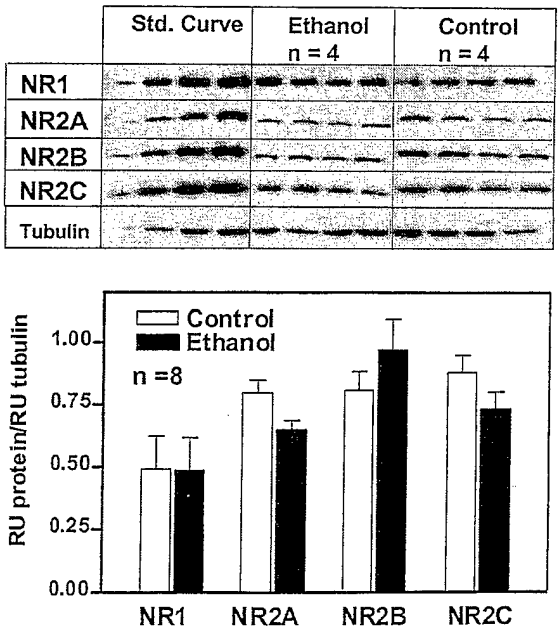

Fig. 2. Shown in the upper panel are samples of Western immunoblots of hippocampal homogenates from ethanol-treated and control rats that were probed with the indicated anti-NMDA receptor subunit antibodies. As illustrated, a standard curve was generated with a single batch of sample in all cases. Values from this standard curve were used to calculate relative units (RU) of protein concentration. Relative units of tubulin levels were also determined and used to normalize the results (see "Methods" for details). Shown in the lower panel is the summary of the results. Values were not found to be statistically different by two-way ANOVA ($p > 0.16$).

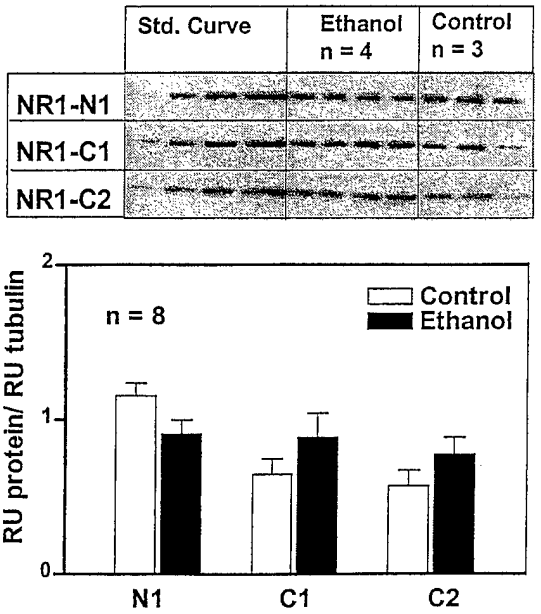

Fig. 3. Shown in the upper panel are samples of Western immunoblots of hippocampal homogenates from ethanol-treated and control rats that were probed with antibodies that recognize NR1 subunits containing the N1, C1, or C2 cassettes. See the legend for Fig. 2 for more details. Shown in the lower panel is the summary of the results. Values were not found to be statistically different by two-way ANOVA ($p > 0.5$). RU, relative unit.

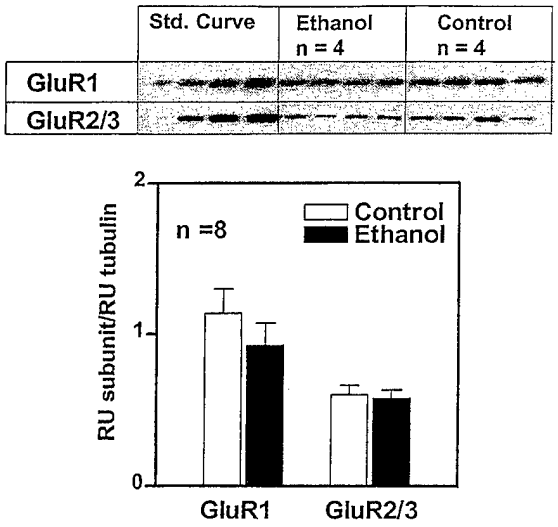

Fig. 4. Shown in the upper panel are samples of Western immunoblots of hippocampal homogenates from ethanol-treated and control rats that were probed with the indicated anti-AMPA receptor subunit antibodies. See the legend for Fig. 2 for more details. Shown in the lower panel is the summary of the results. Values were not found to be statistically different by two-way ANOVA ($p > 0.4$). RU, relative unit.

subunits, we did not detect alterations in the levels of kainate receptor subunit expression in the chronically ethanol-exposed animals (Fig. 5).

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DISCUSSION

In this study we examined the effects of a 16-day ethanol-containing diet on the expression of receptor subunits be-

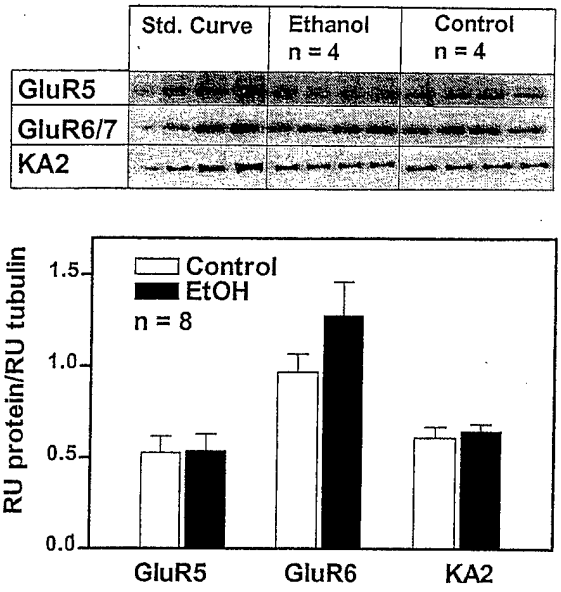

Fig. 5. Shown in the upper panel are samples of Western immunoblots of hippocampal homogenates from ethanol-treated and control rats that were probed with the indicated antikainate receptor subunit antibodies. See the legend for Fig. 2 for more details. Shown in the lower panel is the summary of the results. Values were not found to be statistically different by two-way ANOVA ($p > 0.4$). RU, relative unit.

longing to all three families of glutamate-gated ion channels. We studied a separate group of rats the day after the end of this diet and determined, by assessing both withdrawal scores and the presence of audiogenic seizures, that it clearly results in the development of ethanol dependence. Unexpectedly, we did not find differences in NMDA-R subunit expression in the hippocampus of rats when assessed at the peak of ethanol consumption on day 16 of this diet. Our results are somewhat unexpected because other studies have reported changes in NMDA-R subunit expression after long-term ethanol exposure paradigms. For instance, a recent study by Devaud and Morrow (1999) found an approximately 20% increase in the levels of NR1 and NR2A subunit expression in hippocampal homogenates of male Sprague-Dawley rats exposed to a liquid diet that produced blood ethanol levels in the 35 to 50 mM range and that were killed while still on this diet. Kalluri et al. (1998) found an approximately 35% increase in the levels of NR1, NR2A, and NR2B in the hippocampus of male Sprague-Dawley rats that received 9 to 15 g/kg of ethanol per day by intragastric intubation three times a day for 6 days and that were killed 1 hr after the last dose of ethanol. Trevisan et al. (1994) detected an approximately 65% increase in NR1 immunoreactivity in the hippocampus of male Sprague-Dawley rats that were exposed to a 12-week ethanol-containing liquid diet. These rats had blood ethanol levels of 50 to 65 mM at the time of death. In contrast to these studies, Winkler et al. (1999) found that hippocampal NR1 subunit levels did not change in alcohol-preferring (AA) or alcohol-nonpreferring (ANA) rats exposed to an ethanol-containing diet, which produced maximum blood

ethanol levels of approximately 30 mM, for 30 days. In agreement with the study of Winkler et al., we did not find an effect of long-term ethanol on hippocampal ionotropic glutamate receptor expression; this indicates that changes in NMDA-R subunit expression do not always occur in rats chronically exposed to ethanol.

The effects of chronic ethanol exposure on the expression of alternatively spliced isoforms of the NR1 subunit have recently been studied, with emphasis on messenger RNA (mRNA) levels. Hardy et al. (1999) reported a decrease in the ratio of NR1 mRNA containing the segment encoding for the N1 insert in the cerebral cortex of male Wistar rats exposed to ethanol vapor for 16 days. These rats were killed before withdrawal and had blood ethanol levels of 50 to 100 mM. A more recent in situ hybridization study, also performed with male Wistar rats, detected a decrease in hippocampal mRNA levels of NR1 subunits containing the C2 (NR1-2) insert after administration of 9 to 15 g/kg/day of ethanol for 8 days via intragastric intubation (Darstein et al., 2000). In the study of Winkler et al. (1999) discussed previously, it was found that chronic ethanol exposure of AA and ANA rats did not affect mRNA levels for NR1-1 (contains the C1 and C2 cassettes), NR1-2 (contains the C2 cassette), NR1-3 (contains the C1 cassette), or NR1-4 (does not contain the C1 or C2 cassettes). It is interesting that Western blot analyses revealed that the signal obtained with an anti–NR1-3/1-4 antibody significantly increased in ethanol-exposed AA rats, but not in ANA rats (Winkler et al., 1999). In contrast to these studies, we found no effects of long-term ethanol exposure of male Sprague-Dawley rats on the protein expression levels of hippocampal NR1 subunits containing the N1, C1, and C2 inserts. However, it is difficult to compare our study with the work of Hardy et al. (1999) and Darstein et al. (2000) because these studies did not examine the protein expression levels of NR1 subunit splice variants. Moreover, it is also difficult to compare our results with those of Winkler et al. (1999) because they used antibodies that cannot distinguish among subunits containing the C1 cassette only (NR1-3) or subunits lacking the C1 and C2 cassettes (NR1-4). Therefore, it would be important to determine whether exposure to chronic exposure paradigms such as the one used by Winkler et al. (1999) produces changes in the protein levels of NR1 splice variants when assessed with more specific antibodies.

An important finding of our study is that we did not detect any differences in the expression of GluR1 and GluR2/3 subunits. These results are in agreement with those of Trevisan et al. (1994), who reported that a 12-week exposure to alcohol did not change hippocampal levels of either GluR1 or GluR2 subunits in the hippocampus of male Sprague-Dawley rats. We also evaluated expression of GluR5, GluR6/7, and KA2 kainate receptor subunits and found it to be unaltered after 16 days of ethanol exposure. To the best of our knowledge, this is the first study on the effects of long-term ethanol exposure of rats on kainate receptor subunit expression. Therefore, it would also be important to corroborate our findings by using different ethanol-exposure paradigms than the one used in this study.

It is important to compare the findings of our study with those of some investigations on the long-term effects of ethanol exposure of cultured neurons. Ticku and collaborators have reported that exposure of cultured cortical neurons to 50 to 75 mM ethanol for 5 days results in an increase in [^{3}H]MK-801 binding and also in up-regulation of NR1 and NR2B mRNA and protein subunit levels (Follesa and Ticku, 1995, 1996; Hu and Ticku, 1995; Hu et al., 1996; Kumari and Ticku, 1998). Chandler et al. (1997) reported that ethanol exposure (100 mM for 4 days) of cultured cortical neurons grown in the presence of 2 mM glutamine did not change [^{125}I]MK-801 binding or the expression levels of NR1, NR2A, and NR2B subunits. However, this treatment increased NMDA-stimulated nitric oxide production. A subsequent study by the same group of investigators, which was also performed with cultured cortical neurons exposed to 100 mM ethanol for 4 days, detected an increase in levels of NR1, NR2A, NR2B, GluR1, and GluR2/3 subunits, but not of GluR6/7 subunits (Chandler et al., 1999). It is important to note that these ethanol-induced changes in subunit expression were highly dependent on the culture conditions, because these were observed in cells grown in low (0.1 mM), but not high (2 mM), glutamine. Hoffman et al. (1995, 1996) reported a 47% increase in [^{3}H]MK-801 binding and a 20% increase in NR1 and a 30% decrease in NR2A subunit levels in cultured cerebellar granule neurons exposed to 100 mM ethanol for 2 to 4 days. In contrast, Cebere et al. (1999) reported that exposure of cultured cerebellar granule neurons to 50 to 100 mM ethanol for 3 days did not change [^{3}H]MK-801 binding or expression of NR1 splice variants and NR2A, NR2B, and NR2C subunits. More recently, Kumari (2001) found that exposure of cultured cortical neurons to 50 mM ethanol for 5 days induced a decrease in the mRNA and protein levels of splice variants containing the N1 cassette (NR1–3b and NR1–4b) and an increase in the protein expression levels of splice variants lacking this cassette (NR1–3a and NR1–4a). As in the case of the animal studies mentioned previously, the results of these studies clearly indicate that the effects of long-term ethanol exposure of cultured neurons on ionotropic glutamate receptor subunit expression are also variable and highly dependent on the experimental conditions used by different investigators. However, an important conclusion that can be derived from these in vitro studies is that chronic ethanol exposure does not always result in compensatory changes in ionotropic glutamate receptor subunit expression; this is in agreement with the findings of our study.

In conclusion, we found that exposure of rats to a 16-day ethanol exposure paradigm, which clearly results in the development of ethanol dependence, is not associated with changes in the hippocampal expression of subunits belong-

ing to all of the three major families of ionotropic glutamate receptors. Our findings are in agreement with those of Rudolph et al. (1997), who reported that robust changes in ligand binding to glutamate receptors in the adult rat brain do not occur with several chronic ethanol treatment protocols, including a liquid diet similar to the one used in this study. Therefore, maladaptive changes in brain ionotropic glutamate receptor levels do not underlie, in all cases, the neurobehavioral consequences of chronic ethanol exposure.

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